Evaluating association and transmission of eight inflammatory genes with Viliuisk encephalomyelitis susceptibility

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Summary

Since the discovery of Viliuisk encephalomyelitis (VE) in 1887, scientists have tried to understand the natural history and aetiology of this endemic neurological disorder among the native Sakha population of Central Siberia. Familial aggregation and segregation analysis suggested a genetic influence on VE incidence. However, recent studies have implicated an unknown virus, possibly from the alpha herpesvirus family, as a possible cause for this disease. As VE is a neurological disease characterized by the inflammatory reactions systematically observed in the spinocerebellar fluid and in the brain tissue of deceased patients, we examined 17 single nucleotide polymorphisms (SNPs) across seven inflammation-related candidate gene regions, including chemokine receptors type 2 and 5 (*CCR2/CCR5*), interferon-γ (*IFN-γ*), interleukin-4 (*IL-4*), *IL-6*, *IL-10*, stromal cell-derived factor (*SDF*) and chemokine regulated upon activation, normal T-cell expressed and presumably secreted (*RANTES*). Our main objective was to analyse the degree of genetic association between VE and candidate genes that have been previously implicated in other inflammatory diseases. Samples were collected from 83 affected families comprising 88 verified VE cases, 156 family members, and an additional 69 unrelated, unaffected inhabitants of the same geographical area. This collection included substantially all of the cases that are currently on the VE Registry. The experimental design included both case–control and transmission/disequilibrium test (TDT)-based familial association analyses. None of 17 SNPs analysed was significantly associated with VE occurrence. Exclusion of these eight genes based on the lack of association has important implications for identifying the disease agent, as well as prescribing therapy and understanding Viliuisk encephalomyelitis.

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Introduction

Viliuisk encephalomyelitis (VE), found almost exclusively among the Sakha people of Siberia, is a progressive neurological disorder with a fatal outcome. This subacute meningo-encephalitis progresses in many cases to a prolonged pan-encephalitic syndrome in which death usually occurs within 1–10 years, although some patients survive to a steady state of global dementia, rigidity, and severe spasticity that may last for over 20 years.

VE is different from arboviral encephalitides, such as the well-known tick-borne encephalitis, Eastern equine encephalitis, or West Nile encephalitis, because of its low communicability and a long incubation period between the initial contact and first symptoms. In addition, in VE cases the neuropathological examination reveals inflamed meninges, randomly scattered multiple small necrotic foci surrounded by inflammatory infiltrates in the cerebral cortex and other grey matter areas (McLean *et al*., 1997; Vladimirtsev *et al*., 2000). Epidemiological studies as well as the presence of oligo-clonal cerebro-spinal fluid (CSF) bands indicate the role of an infectious agent; on the other hand, household, family and ethnic clustering is consistent with a hereditary component (Goldfarb & Gajdusek, 1992; Osakovsky & Sivtseva, 2000; Green *et al*., 2003).

VE was discovered in Viliui, a small region of the Sakha Republic in north-western Asia, in the Siberian part of the Russian Federation. The Sakha, also known by their Russian names as Iakut or Yakut, live in the basin of the Lena River, between Lake Baikal and the Arctic Ocean. VE disease has been known here for at least a hundred years, and the native Sakha called it 'bokhoror', meaning 'stiffness'. Historically, VE has been restricted to the area along the Viliui river, a large tributary of the Lena. However, due to population movements in recent years, especially after World War II, VE has expanded to a larger territory that encompasses most of southern Sakha (Stone, 2002), a very large, but sparsely populated area comparable in size to the Eastern United States. Communication and contact between previously isolated Siberian villages have increased dramatically, and it has been suggested that the increased

contact with the outside world may lead to greater health risks and the worldwide spread of this disease (Garruto *et al*., 1999). Expressing its concern over the spread of VE, the World Health Organization (WHO) organized a collaborative research effort among international collaborators and encouraged molecular and genetic studies to search for VE susceptibility genes (WHO, 1998).

While infections are mediated by migrants, indicating evidence for horizontal transmission in a setting of a long, intimate contact, a detailed examination supported a genetic predisposition to the disease. The strongest support for a genetic predisposition to VE comes from the observed familial clustering of the disease; familial hereditary predisposition for VE is as high as 22 to 28%. These results indicate that heredity might be involved in VE aetiology (Goldfarb & Gajdusek, 1992; Goldfarb *et al*., 1998). Goldfarb & Gajdusek (1992) also examined pedigrees by segregation analysis and demonstrated that VE had a significant heritability in the affected families; however, its transmission is incompatible with the Mendelian mode of inheritance, suggesting multifactorial origins of the disorder.

It is also evident that VE is still restricted to the ethnically distinct Sakha populations, but is gradually spreading to surrounding areas (Goldfarb & Gajdusek, 1992). According to the official census (Alekseev, 2000), 38% of the total population of the Sakha Republic of one million is of Sakha nationality, while ethnic Russians constitute 54% of the population. The prevalence of VE in the ethnic Sakha is 0.025% overall and could be as high as 1.5% among the natives of some areas along the Viliui River, whereas there are no confirmed cases of VE in the non-Sakha populations living in the same areas (Alekseev, 2000). Thus, VE is a significant cause of morbidity and mortality in the Sakha Republic, especially in some regions of the Middle Viliui area, where nearly 1.5% of the adult Sakha populations die of VE (Alekseev, 2000).

The cause of VE still remains unknown. Although VE is fatal in susceptible individuals, the fact that most individuals are not infected within such a tightly knit society as the Sakha or even within VE-prone families suggested avenues for investigating host susceptibility to the disease development. Earlier research in which VE blood, serum, CSF and brain tissue were inoculated into cell cultures failed to unveil the causative agent (Goldfarb & Gajdusek, 1992; Goldfarb *et al*., 2000). More recent experiments have suggested that VE could be associated with an intrathecal synthesis of immunoglobulin G (IgG), which shows immunoreactivity against herpes simplex I and II antigens but not other known viral antigens in VE patients (Green, 2000; Green *et al*., 2003). This led some researchers to conclude that antigenic components of the VE virus may be represented on the cellular membrane by an autoimmune allele of the immunity gene and may serve as a receptor for a pathogenic action of antibodies to its own tissue (Osakovsky & Sivtseva, 2000). The autoimmune reaction would trigger inflammatory genes and lead to neuronal tissue destruction. However, there is no direct evidence that demonstrates this postulated mechanism.

Many genes have been implicated in the initiation and conduction of the host immune response. An array of such genes are involved in the progression of human immunodeficiency virus (HIV) infection to AIDS as well as in other immune disorders (Hill, 1998; O'Brien & Moore, 2000; O'Brien *et al*., 2000; Chinen & Shearer, 2002; Thomson *et al*., 2002). In this study, we have tested for association with some well-characterized genes, including chemokine receptors type 2 and 5 (*CCR2/CCR5*), interferon-γ (*IFN-γ*), interleukin-4 (*IL-4*), *IL-6*, *IL-10*, stromal cell-derived factor (*SDF*) and chemokine regulated upon activation, normal T-cell expressed and presumably secreted (*RANTES*). These loci were chosen as part of our larger efforts in candidate gene testing, and several of them have been associated with HIV-AIDS progression (O'Brien *et al*., 2000). Thus, we believed that these loci might be more likely to be associated with other immune diseases including VE. We employed a case–control design for the individual alleles, genotypes and the reconstructed haplotypes, where possible, of these genes, using unrelated controls from the same geographic locations. In addition, with the same set of cases, we conducted a familial pedigree study, using both allelic and haplotypic data to test for significant transmission disequilibrium of the inflammatory gene polymorphisms and VE.

Materials and methods

Subjects and study design

Our study used blood samples from 313 individuals from four different regions in the Sakha Republic. Of these, 88 were individuals with different stages of VE progression and 156 were their relatives. In addition, we obtained samples from 69 healthy individuals who were not related to the VE patients (controls), and represented the general populations in the same areas where the VE samples were collected. Eighty-eight verified VE cases, ascertained by strict diagnostic criteria (Goldfarb & Gajdusek, 1992; Vladimirtsev *et al*., 2000), were identified, and 83 affected family pedigrees were tested using the transmission/disequilibrium test (TDT) (Clayton, 1999). The distribution of VE patients was as follows: 33 from the East-West Viliui area, 17 from the Lena-Aldan area, 17 from the North Viliui area, and 21 from the South Viliui area (Fig. 1). The distribution of the control samples was 26, 20, 15 and 8, respectively. We also obtained samples from 43, 36, 28 and 49 individuals closely related to the VE patients in the same areas.

Inflammatory genes

We used 17 single nucleotide polymorphisms (SNPs) distributed across eight genes that are well described and known for their involvement in autoimmune disorders (Fiorentino *et al*., 1989; Moore *et al*., 1990, 2001; Rode *et al*., 1993; Schall & Bacon, 1994; Hendel *et al*., 1998; Martin *et al*., 1998; Ward & Westwick, 1998; Shin *et al*., 2000; Chesler & Reiss, 2002; Haddad, 2002) (see Table 2 below). We investigated whether there were associations

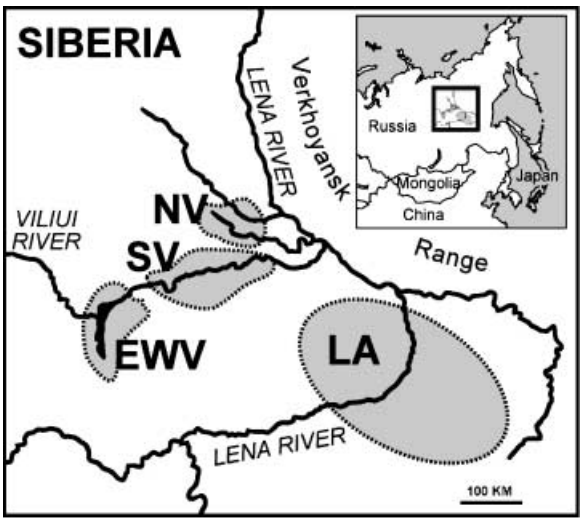

Figure 1. Distribution of Viliuisk encephalomyelitis (VE) in Siberia. Case and control samples came from four different regions known to contain VE: EWV (East-West Viliui Region), SV (South Viliui Region), NVC (North Viliui Region), and LA (Lena Aldan Region).

between these polymorphic alleles, as well as the haplotypes identified by these alleles, and VE susceptibility.

DNA extraction and SNP genotyping

Using the standard Qiagen protocol (Qiagen, Hilden, Germany), DNA was extracted from whole blood collected from these individuals. Typing of the SNP polymorphisms was performed by polymerase chain reaction (PCR) on a Perkin Elmer Thermal Cycler 9700 (Perkin Elmer, Foster City, CA). To detect SNP polymorphisms at these loci, we employed a single-base extension protocol from Applied Biosystems (Foster City, CA) using a fluorescent ddNTP length-modified single base extension (Li *et al.*, 1999). The 17 loci were multiplexed on two different panels, allowing high-throughput data generation (Kwok, 2001). Genotyping of SNPs was simplified with automation, using Genemapper software (Applied Biosystems) to avoid the errors associated with manual processing (Bishop *et al.*, 2002).

Verification of family pedigrees

To analyse family structures with the TDT test, we needed to make sure that the self-reported family genealogies were correct. We genotyped all of the samples with a set of eight pentanucleotide short tandem repeats (STR) markers known to have significant power (matching probability = 3.8×10^{-11}) to differentiate between any two individuals. When an impossible combination of STR alleles was identified, DNA of members of the suspected family was singled out and re-amplified two times to rule out genotyping error. Two of the affected families failed to confirm the self-reported pedigrees, so these families were excluded from further analysis.

Statistical analysis

The statistical analysis for this study was performed using SAS/Genetics software (SAS, 2003), or Transmit 2.1 (Clayton, 1999). Several SNPs that deviated from the Hardy–Weinberg equilibrium in the exact test (Weir, 1996) were excluded from the analysis. We also did not analyse loci that lacked variation.

We tested both the allelic and genotypic associations in the affected individuals vs. unaffected healthy controls from the same areas in a case–control study. When several SNPs were available on the same gene, we computed the haplotypes to test for haplotype association with the disease transmission. Phase 1.01 by Stephens *et al.* (2001) was used to resolve the ambiguous haplotypes in controls without the parent genotypes. We used Fisher's exact test to calculate the significance of the difference in allele and haplotype frequencies both in the overall sample and in each of the four regional populations.

Overall, TDT represents the degree of deviation from the expected 50% transmission probability of each of the parental alleles to the affected offspring (Spielman *et al.*, 1993; Spielman & Ewens, 1996; Clayton, 1999). We applied the TDT to check for the association between the transmission of SNPs in affected families and the occurrence of VE. Two of the TDTs available in the SAS/Genetics module (SAS, 2003), S-TDT and RC-TDT, were applied to the each of the individual loci to test for the transmission disequilibrium in the VE-affected families.

To reconstruct parental haplotypes with the familial haplotype TDT, we used the Transmit 2.1 algorithm. In families with missing parents, the algorithm calculates transmission probabilities by using the genotype of siblings to estimate the genotype of the missing parent (Clayton, 1999).

Results

All of the inflammatory gene polymorphisms included in the analysis complied with the expectations of the Hardy–Weinberg equilibrium (Table 1), which were also met in each of the four populations (data not shown). Common allele frequencies in cases and controls, as well as heterozygosities and the significance values for the Hardy–Weinberg tests for the population of control individuals, are shown in Table 1.

In the case–control study, we tested allelic and genotypic associations of VE with both the alleles and genotypes of the inflammatory polymorphisms in patients vs. the unrelated healthy controls who came from the same areas as the patients. There were no significant associations of any of the inflammatory gene polymorphisms in any of the allelic tests (Table 2). Similarly, there were no significant associations with VE and the Bayesian-reconstructed haplotypes for *CCR2/CCR5*, *IL-4*, *IL-6* or *IL-10*.

In the transmission disequilibrium study, we used three different approaches to test these inflammatory gene polymorphisms. First, we used the sibling transmission/disequilibrium test (S-TDT) (Spielman & Ewens, 1996), a

Table 1. Locus names, allele names, common allele frequencies, observed heterozygosities (H) and Hardy–Weinberg (HW) equilibrium significance values for the allele distributions of inflammatory gene polymorphisms in Viliuisk encephalomyelitis controls, along with common allele frequencies in cases

				Cases (n = 88)	Controls (n = 69)		
Gene	SNP	NCBI name	Alleles	Allele frequency	Allele frequency	H	HW *P*-value†
CCR2/	V64I	rs1799864	G/a	0.58	0.58	0.48	0.88
CCR5	59353	rs1799988	C/t	0.57	0.55	0.38	0.07
	59356	—	G/a	0.99	1.00	0.00	—
	59402	rs1800023	T/c	0.80	0.75	0.32	0.24
	Δ32	rs333	+/?32	0.99	1.00	0.00	—
IFN-γ	5299	—	A/g	0.87	0.79	0.32	0.81
IL-10	4467	rs3024495	G/a	0.94	0.98	0.04	0.85
	5016	rs3024496	T/c	0.93	0.96	0.09	0.70
	4299	rs3024498	A/g	0.99	0.99	0.03	0.90
	1082	rs1800896	A/g	0.91	0.93	0.12	0.26
	592	rs1800872	A/c	0.62	0.70	0.37	0.33
IL-4	–1098	rs2243248	T/g	0.94	0.96	0.09	0.70
	144	—	C/t	0.98	0.99	0.02	0.95
IL-6	205	rs2069849	C/t	0.98	0.98	0.04	0.85
	3635	rs1880243	A/c	0.71	0.70	0.48	0.25
	4731	rs1554606	T/g	0.97	0.95	0.11	0.65
	6021	rs2069849	T/c	0.99	0.97	0.06	0.80
	9699	rs2069845	G/a	0.97	0.95	0.10	0.66
RANTES	403A	rs2107538	G/a	0.57	0.51	0.47	0.60
SDF1	3′A	rs1801157	G/a	0.85	0.87	0.16	0.03

NCBI, National Centre for Biotechnology Information.
The most common allele is given in capitals for the SNP.
† *P*-values are from the exact test (Weir, 1996).

test that can be applied to families with at least one affected offspring, and in which not all siblings have the same genotype. None of the examined SNPs showed significant transmission disequilibrium (Table 3). Next, we applied the reconstruction-combined transmission/disequilibrium test (RC–TDT) test (Knapp, 1999a) to test for significant transmission disequilibrium in the same data. RC–TDT reconstructs missing parental haplotypes when possible from pedigrees in order to use more families. None of the tested loci was significantly associated with the transmission of VE in families of the infected individuals (Table 2). Finally, we applied a haplotypic TDT test to the haplotypes of *CCR2/CCR5*, *IL-4*, *IL-6* and *IL-10* VE using Transmit 2.1 (Clayton, 1999). The observed and expected values of the reconstructed haplotypes and their corresponding *P*-values (Table 3) showed no significant associations with the transmission of any of these genes.

Discussion

The aetiology of VE is unknown, but the inflamed meninges, micronucleic foci surrounded by the inflammatory cells and perivascular infiltrates in the brain parenchyma suggested that long-term persistent inflammation in the brain is part of the pathogenesis of this disease (Goldfarb & Gajdusek, 1992; McLean *et al.*, 1997). It has been proposed that inflammation originates from the ongoing viral-mediated process in the nervous system, a process similar to that for herpes simplex encephalitis (Lellouch-Tubiana *et al.*, 2000). The working hypothesis in our study was based on the possibility that cross-reactivity to host antigenic components based on an antigenic response to the putative VE virus may be a pathogenic mechanism of neuronal tissue damage (Osakovsky & Sivtseva, 2000). In this hypothesized mechanism and related mechanisms, the autoimmune reaction would ultimately trigger inflammatory genes and lead to tissue destruction. The inflammatory process is most active in the subacute and chronically progressive phases of illness, during which cellularity in the brain tissue increases (T lymphocytes, some B lymphocytes, activated macrophages and microglia) and the intrathecal production of IgG is most active. These phenomena subside in the end-stage disease corresponding to the 'burnt-out' condition characterized pathologically by fibrosis and atrophy with minimal residual inflammation (Green *et al.*, 2003). Therefore, inflammatory gene polymorphisms could have some effect on VE outcome. We tested this hypothesis by studying an association of a set of gene polymorphisms that had earlier been implicated in the initiation and regulation of the host immune response and VE.

IL-10 is a multifunctional cytokine with diverse effects on most hemopoietic cells (Moore *et al.*, 2001). It was first recognized for its ability to inhibit activation and

Table 2. Significance values for the individual loci case–control and transmission/disequilibrium (TDT) tests for associations between Viliuisk encephalomyelitis (VE) and selected inflammatory gene polymorphisms

		P-values[a]			
		Case–control		TDT[b]	
Genes	Loci	Allelic	Genotypic	S-TDT	RC-TDT
CCR2/[c]	V64I	0.96	0.51	0.91	0.91
CCR5	59353	0.70	0.89	0.32	0.32
	59356	0.23	0.69	0.51	0.52
	59402	0.30	0.62	—	—
	Δ32	0.21	0.46	0.51	0.41
	haplotypes[d]	0.78	0.99		
IFN-γ	5299	0.09	0.24	0.08	0.09
IL-10	4467	0.13	0.29	0.87	1.00
	5016	0.37	0.65	0.23	0.23
	4299	0.42	0.72	0.69	0.69
	1082	0.58	0.29	0.32	0.32
	592	0.14	0.38	0.87	0.87
	haplotypes	0.15	0.98		
IL-4	–1098	0.47	0.77	0.12	0.08
	144	0.32	0.59	0.72	0.81
	haplotypes	0.96	0.99		
IL-6	205	0.97	0.99	0.16	0.32
	3635	0.86	0.74	0.91	0.82
	4731	0.42	0.71	0.16	0.32
	6021	0.26	0.52	—	—
	9699	0.45	0.74	0.16	0.32
	haplotypes	0.24	0.66		
RANTES	403A	0.32	0.46	0.87	0.63
SDF1	3′A	0.57	0.03	0.16	0.16

[a] *P*-values represent the significance of the exact tests (Weir, 1996).
[b] S-TDT (Spielman & Ewens, 1996) can be applied to families with at least one affected offspring, in which not all siblings have the same genotype. RC-TDT reconstructs missing parental haplotypes when possible to use more families (Knapp, 1999a).
[c] *CCR2* and *CCR5* loci are combined on the same haplotype (Martin *et al.*, 1998).
[d] Haplotypes were reconstructed using Phase 2.1 (Stephens *et al.*, 2001) and tested for association with VE using the SAS/Genetics FAMILY procedure (SAS, 2003).

effector function of T cells, monocytes and macrophages (Fiorentino *et al.*, 1989). The main routine function of IL-10 appears to be limitation and termination of inflammatory responses (Moore *et al.*, 2001). IL-10 has been implicated in the genetic restriction of HIV-1 pathogenesis to AIDS (Shin *et al.*, 2000) and has related homologues in a number of viral genomes (Moore *et al.*, 1990; Rode *et al.*, 1993). This again illustrates the important role of this gene in regulation of immune and inflammatory responses.

IL-4 was first described as a cofactor in the proliferation of resting B cells. It was also described as a T-cell factor that induced B-cell differentiation into plasma cells. IL-4 also plays a major role in T-cell development. It is thought to be influential in promoting differentiation of T helper cells into Th2 cells during an immune response (Haddad, 2002). IL-6, initially identified as a B-cell differentiation factor, is known to be a multifunctional cytokine that regulates the immune response, inflammation, and the acute phase response (Ishihara & Hirano, 2002). Both clinical data and animal models suggest that IL-6 plays a critical role in the pathogenesis of autoimmune diseases.

IFN-γ is a marker of Th1 CD4, CD8 and natural killer cells and is involved in the regulation of nearly all phases of immune and inflammatory responses (Haddad, 2002). It is also a critical antiviral mediator that is central to the elimination of viruses from the central nervous system. IFN-γ has been studied in the pathogenesis of herpes simplex virus (Reiss *et al.*, 2002). IFN-γ, as well as inflammatory cytokines (IL-12 and TNF), acts to elicit the innate antiviral responses in neurons (Chesler & Reiss, 2002; Reiss *et al.*, 2002).

Chemokines (SDF1, RANTES) and their receptors (CCR2/CCR5) are well known for their chemotactic effects on a variety of leukocytes (Schall & Bacon, 1994). This group of receptors has attracted a lot of attention because of their involvement in primary HIV-1 infection (Hendel *et al.*, 1998; Martin *et al.*, 1998; O'Brien & Moore, 2000). Chemokines have many overlapping functions

Table 3. Transmission–disequilibrium tests for associations between Viliuisk encephalomyelitis and the haplotypes of selected inflammatory gene polymorphisms from Transmit 2.5 (Clayton, 1999)

Genes	Haplotypes[a]	Observed	Expected	χ^2	*P*-values
CCR2/CCR5	GTACG[b]	1.15	0.71	0.78	0.38
	GCGCA	1.01	0.61	0.66	0.42
	ACGCG	0.21	1.39	2.85	0.09
	GTGCG	14.50	14.42	0.00	0.97
	GTATG	1.05	0.66	0.65	0.42
	ACGTG	24.43	26.50	0.73	0.39
	GCGTG	11.78	14.49	1.69	0.19
	ATGTG	2.36	1.53	1.43	0.23
	GTGTG	19.52	15.69	3.62	0.06
	Overall[c]				0.33
IL-10	AAACA	45.72	49.62	2.34	0.13
	ACACA	28.28	25.66	1.40	0.24
	GCACA	3.00	2.45	0.32	0.58
	GCGTA	6.00	4.43	1.63	0.20
	GCATG	1.00	1.84	0.98	0.32
	Overall				0.31
IL-4	GC	71.93	72.84	0.27	0.60
	GT	3.07	1.91	1.90	0.17
	TC	6.07	6.67	0.18	0.68
	TT	0.93	0.58	0.60	0.44
	Overall				0.45
IL-6	CAGA	4.00	3.14	0.66	0.42
	TAGA	1.00	0.62	0.61	0.44
	TATG	55.00	55.79	0.10	0.75
	TCTG	24.00	24.46	0.04	0.84
	Overall				0.73

[a] These haplotypes are composed of the following SNPs: –1082, –592, 4467, 5016 and 5351 for *IL-10*; CCR2 64I and CCR5?32, 59353, 59356 and 59402 for the combined haplotype of *CCR2* and *CCR5*; 205, 3635, 4731 and 9699 for *IL-6*, and –1098 and –144 for *IL-4*.

[b] Individual haplotypes are tested in a series of 1 degree of freedom (d.f.) tests.

[c] The overall tests for association are based on the *n* haplotypes with transmission data with (*n* – 1) d.f.

and are produced by a variety of cell types during the inflammatory response (Ward & Westwick, 1998).

However, none of the polymorphisms selected from the seven inflammatory gene regions demonstrated significant degrees of association with VE in case–control tests involving the individual SNPs, as well as the reconstructed haplotypes located in these genes. Similarly, TDT test results showed no positive association between VE and transmission of selected alleles or haplotypes. This result was perhaps surprising, as recent studies had strongly suggested the involvement of inflammatory mechanisms in VE pathology. In part, the lack of significant association may be due to an interaction between factors, both genetic and environmental, that may be involved in VE. However, addressing these interactions would require a very large study, and there are only a limited number of samples available.

Unfortunately, while we were able to examine an exhaustive set of samples representing nearly all of the available patients, we were still limited in our ability to detect a complex genetic association with a set of genetic markers. The total number of diseased individuals required to detect a dominant susceptibility allele with a power of $\beta = 0.80$ in the case–control studies with $\alpha = 0.05$ and frequency of the disease allele $P = 0.1$ is between 46 and 92, according to different estimates (Jackson *et al.*, 2002). Similarly, S-TDT and RC-TDT tests described in the literature demonstrate that a larger sample size is also needed in order to gain the 0.80 power needed to detect the transmission disequilibrium of a particular allele associated with disease (Knapp, 1999a,b). We recognize and clearly acknowledge the problem of lack of statistical power. However, the ability to detect associations depends on their strength. Given that there were reasons to anticipate a strong association between VE occurrence and inflammatory gene polymorphisms based on previous research (Green *et al.*, 2003), we optimistically hoped to find a strong signal at one of these loci. However, further work on these genes and others could be fruitful with larger sample sizes of patients and more family pedigrees.

There are more than eight inflammatory genes and there are additional SNPs in these genes. Some of the genes we tested had sufficient numbers of SNPs to detect the major haplotypes (Gabriel *et al.*, 2002), and others, especially *IFN-γ*, *RANTES*, and *SDF1*, may need to be examined with a higher density of markers. On the other hand, additional gene regions, such as those recently implicated in multiple sclerosis and other T-cell-mediated autoimmune diseases (Bomprezzi *et al.*, 2003), can be suggested for future studies.

The results of the case–control and transmission disequilibrium studies showed no positive association between VE and inflammatory gene polymorphisms. However, this does not rule out the possibility that further work on this set of genes might be fruitful. A larger sample will be needed to achieve the power desired for a conclusive result. This collection of individuals and our analytic approach have laid the foundation for discovering the natural human variants that can contribute to the identification of the genetic aetiology of VE.

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